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Michelle Henkel

MICHELLE HENKEL
TEAM LEADER EXAMINATION
SUPPORT AND SALES

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PROVISIONAL SPECIFICATION

Invention Title:

Random Drift Mutagenesis

The invention is described in the following statement:

Technical Field

The present invention is directed to methods for producing mutant genes and mutant gene products.

5 Background Art

There are many situations such as medical, veterinary and industrial applications, where mutant gene products are desirable. Microorganisms can produce a great variety of proteins, particularly enzymes, which can be used in many situations. Many of these proteins, however, have characteristics that are
10 not ideal for subsequent use and therefore it is desirable to obtain mutants with altered activity or functions. There are a number of methods and techniques presently in use which can generate mutant genes and corresponding mutant gene products.

Stemmer (Stemmer, W. P. C. Rapid evolution of a protein *in vitro* by DNA
15 shuffling. *Nature* **370**, 389 - 391, 1994.; Stemmer, W. P. C. DNA shuffling by random fragmentation and reassembly: *in vitro* recombination for molecular evolution. *Proc. Natl. Acad. USA* **91**, 10747 - 10751, 1994) has discussed the most effective methods to search sequence space *in vitro* to yield the greatest diversity of protein variants. Until recently, the most popular methods of creating
20 combinatorial libraries are recursive strategies that seek to evolve sequences by the addition of point mutations. For *in vitro* evolution, inclusion of recombinant polymerase chain reaction PCR (gene shuffling) offers practical and theoretical advantages over simple recursive mutagenesis methods. It will rapidly fine tune the mutational load in several parts of the protein by recombining point mutations
25 and wild-type sequences. The technique (and its variations) have been used to enhance enzyme activity, substrate specificity and stability. Family shuffling is usually achieved by fragmentation of the genes to be shuffled followed by PCR. This method relies on homologous recombination during the PCR reassembly step. Most methods require relatively high levels of sequence similarity between
30 the genes to be shuffled as 'cross-over points' appear to occur in these regions.

If sequence similarity is low between the input genes, the majority products tend to be the reassembled parental genes and extensive searches

need to be carried out to find recombinants (Kikuchi, M., Ohnishi, K. & Harayama, S. Novel family shuffling methods for the in vitro evolution of enzymes. *Gene* **236**, 159 - 167, 1999; Ostermeier, M., Shim, J. H. & Benkovic, S. J. A combinatorial approach to hybrid enzymes independent of DNA homology. *Nat. Biotechnol.* **17**, 1205 - 1209, 1999.). Kichuchi et al (1999) have reported on methods for gene shuffling that make use of unique restriction enzyme sites in the sequences of the parental molecules and following cleavage, several PCR steps were carried out to amplify the recombinant genes, a process that allowed hybrid genes to be formed at high frequency. An entirely different procedure was proposed by Ostermeier et al (1999) that allowed the preparation of combinatorial fusion libraries by progressive truncation of coding sequences of the two parental sequences followed by ligation of the fragments and selection for enzyme activity. Either parent can be used to provide 5' sequence for the hybrid gene. This procedure, termed iterative truncation for the creation of hybrid enzymes (ITCHY), can accommodate recombination between genes with as little as 50% sequence similarity and was found to give a wider range of crossovers compared with standard gene shuffling techniques.

The present applicant recently isolated a gene coding for a thermophilic beta-xylanase that had improved performance in the bleaching of paper pulp. It was desired to investigate the possibility of obtaining mutant derivatives that had enhanced stability and an altered pH optimum. Experiments using error-prone PCR and mis-incorporation mutagenesis followed by gene shuffling allowed the identification of mutant genes that coded for a limited sample of the variations in sequence space but required extensive screening for their identification. Gene shuffling following DNaseI fragmentation of related genes (family shuffling) overwhelmingly gave wild type parental sequences as the major products. After several trials of methods designed to reduce the background, a technique was devised that allows shuffling of genes that differ widely in sequence similarity and G:C content and greatly reduces the appearance of wild type genes. Furthermore, the primer extension conditions may be modified to bias the resulting progeny genes towards any one (or more) of the parental input genes. The present applicant termed this procedure Degenerate Oligonucleotide Gene Shuffling (DOGS) which is described in WO 02/18629.

Although there are many options available to generate mutants, those methods do not necessarily result in useful products or may be too time consuming or unsuccessful. The present inventors have now developed a new method capable of producing potentially many different functional mutants.

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Disclosure of Invention

In a first aspect, the present invention provides a method for producing a mutant gene encoding a functional gene product, the method comprising:

- (a) introducing one or more mutations into a gene to form a mutated gene;
- 10 (b) providing the mutated gene to a host microorganism;
- (c) culturing the host microorganism containing the mutated gene under conditions which allow expression of the mutant gene product; and
- (d) selecting a host microorganism capable of producing a functional gene product from the mutated gene.

15

In a preferred form, the method comprises:

- (a) introducing one or mutations into a gene to form a plurality of mutated genes;
- (b) providing the mutated genes to host microorganisms;
- (c) culturing the host microorganisms containing the mutated genes under
- 20 conditions which allow expression of the mutant gene products; and
- (d) selecting host microorganisms capable of producing a functional gene product from a mutated gene.

In a preferred form, the method further comprises:

- (e) obtaining a combined pool of mutated genes encoding functional gene
- 25 products from the microorganisms in step (d) and repeating steps (a) to (d) to form a library of microorganisms containing a plurality of mutant genes capable of being expressed and producing functional gene products.

In another preferred form, the method further comprises:

(f) screening the library of microorganisms to obtain a mutant gene capable of expressing a mutant gene product with an altered or desired activity, function, or characteristic.

Preferably, step (a) is carried out by misincorporation mutagenesis using the polymerase chain reaction (PCR). It will be appreciated, however, that other methods known to the art to introduce one or mutations into a gene would also be suitable. An example includes, but is not limited to, gene shuffling.

Preferably, step (b) is carried out by ligating the mutant gene into a vector and transforming a bacterium with the vector. Methods of introducing gene(s) into microorganisms are well known to the art and would be applicable for the present invention. The vector may be a plasmid or virus, for example. A microorganism particularly suitable for this step is the bacterium *Escherichia coli*. It will be appreciated that other host microorganisms could also be used for this step.

Preferably, the host microorganisms are cultured under conditions in which the native gene would be expected to be expressed in the particular host microorganism. Liquid culture is particularly suitable for this step as it allows easy retrieval of the cultured microorganisms for subsequent use or analysis. It will be appreciated, however, that any suitable culturing means can be used in this step.

In a further preferred form, the culture conditions result in any host microorganism capable of expressing a functional gene having a detectable characteristic. One preferred form of a detectable characteristic is a selectable phenotype. Examples include colour, size, shape, fluorescence, dependency on a supplied metabolite for growth, enzymatic conversion of a supplied substrate which would allow selection based upon colour or fluorescence. In a more preferred form, the gene encodes an enzyme that can form a fluorometric or chromogenic phenotype or character in a microorganism expressing the gene preferably under appropriate conditions.

In a more preferred form, the gene encodes an enzyme which can form a fluorometric or chromogenic phenotype or character in a microorganism expressing the gene under liquid culture conditions.

The present invention is particularly suitable for high volume screening using a sorting or selecting instrument such as a cell sorter or cytometer. Many different enzymes can act on a substrate to produce a coloured product. When the substrate is acted upon in a cell, the cell may change colour which is then detectable. Accordingly, under the appropriate cultural conditions, microorganisms which express a functional gene product will be coloured and therefore selectable over other microorganisms which do not produce a functional product.

A difference between the present invention and other methods for obtaining mutant genes is that it does not apply any selective pressure or testing for altered activity of the mutant gene products during the process, apart from the retention of function by the mutated gene product.

A further difference between the present invention and other methods for obtaining mutant genes is that it does not select for the removal of neutral mutations or mutations which reduce but do not eliminate the function of the mutated gene product.

The method according to the present invention can be used to produce potentially a large number of different mutant genes encoding functional gene products with potentially large numbers of mutations which can then be tested for altered characteristics or desired qualities or function.

In a preferred form, the gene product is an enzyme. More preferably, the enzyme is capable of acting on a substrate such as X-gal which forms a chromogen upon enzymatic hydrolysis, X-gal being defined as 5-Bromo-4-chloro-3-indolyl -D-galactopyranoside.

In a further preferred form, the gene product substrate should be retained on, or within the cell, in liquid culture.

In a second aspect, the present invention provides a mutant gene capable of producing a mutant gene product produced by the method according to the first aspect of the present invention.

In a third aspect, the present invention provides a method for producing a mutant gene encoding a functional enzyme, the method comprising:

- (a) introducing one or more mutations into a gene encoding the enzyme to form a plurality of mutated genes;
- (b) incorporating the mutated genes into vectors;
- (c) transforming host microorganisms with the vectors;
- 5 (d) culturing the host microorganisms containing the mutated genes under conditions which would allow expression of the corresponding native gene to produce the native enzyme such that the microorganisms express the mutant genes;
- (e) providing a substrate upon which the enzyme can act to produce a
- 10 selectable phenotype in any host bacterium producing a functional enzyme; and
- (f) selecting host microorganisms capable of producing a functional gene product from the mutated gene by sorting bacteria having the selectable phenotype.

In a preferred form, the method further comprises:

- 15 (g) obtaining a pool of mutated genes from the microorganisms in step (f) and repeating steps (a) to (f) to form a library of microorganisms containing a plurality of multiply-mutant genes capable of being expressed and producing functional enzymes.

In another preferred form, the method further comprises:

- 20 (h) screening the library of microorganisms to obtain a mutant gene capable of expressing an enzyme with an altered or desired activity, function, or characteristic.

Preferably, the microorganisms are bacteria, A good bacterium is *Escherichia coli*, although other suitable host bacteria can be used.

- 25 Preferably the sorting is done by flow-cytometry and the bacteria selected by changes in their spectral or fluorescence characteristics due to action of the functional enzyme on the substrate.

- 30 In a fourth aspect, the present invention provides a mutant gene capable of producing a mutant gene product produced by the method according to the third aspect of the present invention.

In a fifth aspect, the present invention provides a method for obtaining a mutant or chimeric gene comprising:

- (a) to (f) carrying out the steps according to the first aspect of the present invention to obtain a mutant gene;
- 5 (g) providing the mutant gene to a host microorganism; and
- (h) carrying out a further mutagenic step to obtain a mutant or chimeric gene.

It will be appreciated that any suitable mutagenic process in step (h) would be suitable. Examples include, but not limited to, gene shuffling or degenerate oligonucleotide gene shuffling.

10 In a preferred embodiment, step (h) comprises:

- (i) assigning one or more segments of the mutant gene based on regions of shared encoded amino acid sequence;
- (ii) amplifying the one or more assigned segments of the gene using a primer having a non-degenerate core based on a segment or template of a gene
15 to be amplified, and the core being flanked by both 5' and 3' ends which, if required, are degenerate; and
- (iii) causing recombination of the one or more amplified segments to form a mutant or chimeric gene.

20 In a sixth aspect, the present invention provides a mutant or chimeric gene produced by the method according to the fifth aspect of the present invention.

In a seventh aspect, the present invention provides a method for obtaining a mutant or chimeric gene encoding an enzyme, the method comprising:

- (a) to (h) carrying out the steps according to the third aspect of the present
25 invention to obtain a mutant gene encoding an enzyme;
- (i) providing the mutant gene to a host microorganism; and
- (j) carrying out a further mutagenic step to obtain a mutant or chimeric gene encoding an enzyme.

It will be appreciated that any suitable mutagenic process in step (j) would be suitable. Examples include, but not limited to, gene shuffling or degenerate oligonucleotide gene shuffling.

In a preferred embodiment, step (j) comprises:

- 5 (i) assigning one or more segments of the mutant gene based on regions of shared encoded amino acid sequence;
- (ii) amplifying the one or more assigned segments of the gene using a primer having a non-degenerate core based on a segment or template of a gene to be amplified, and the core being flanked by both 5' and 3' ends which, if
10 required, are degenerate; and
- (iii) causing recombination of the one or more amplified segments to form a mutant or chimeric gene encoding an enzyme.

In an eighth aspect, the present invention provides a mutant or chimeric gene encoding an enzyme produced by the method according to the seventh
15 aspect of the present invention.

The methods according to the present invention are particularly applicable to the directed evolution of any gene/enzyme capable of hydrolysing a synthetic target substrate with fluorogenic properties.

The substrate could be any indoxyl-linked compound. However, any other
20 chromogenic or fluorogenic substrate that is retained in the cell subsequent to hydrolysis, and can be detected by flow cytometry or other means, would be suitable.

Many indoxyl-linked substrates are commercially available as substrates for many glycosyl hydrolases, including, cellulases, beta-glucosidases, beta-
25 galactosidases, mannosidases, xylanases and beta-xylosidases. Substrates are also available for esterases and/or lipases.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be
30 understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were
 5 common general knowledge in the field relevant to the present invention as it existed in Australia before the priority date of each claim of this application.

In order that the present invention may be more clearly understood, preferred forms will be described with reference to the following drawings and examples.

10

Brief Description of the Drawings

Figure 1. Random Drift flow diagram outlining the basic concept of random drift mutagenesis.

Figure 2. X-sugar detailing the diffusion of substrate-linked indoxyl into
 15 *E. coli*, followed by enzymatic cleavage to form an insoluble chromogenic precipitate within the cell.

Figure 3. Flow figure of cell sorting by flow cytometry based upon expression of BglA phenotype and accumulation of insoluble Indigo within the cells. A. Sorting of *E. coli* cell populations containing either the plasmid
 20 pProEXHTC:BglA or pProEXHTC in the presence or absence of the chromagenic substrate X-gal. Key:

Red: Cells expressing BglA in the presence of X-gal

Blue: Cells expressing BglA in the absence of X-gal

Green: Control cells (vector only) in the presence of X-gal

25 Magenta: Control cells (vector only) in the absence of X-gal

B. Part 1. Overlaid datasets from part A showing the clear delineation between cell populations expressing the BglA⁺ phenotype (red) vs BglA⁻ phenotype (green). Parts 2 and 3. Overlaid datasets from part A showing that presence or absence of X-gal did not contribute to observed effects.

Mode(s) for Carrying Out the Invention

The present inventors have developed a new procedure for achieving directed evolution of proteins, particularly enzymes, which are more suited to novel environments and applications than their natural counterparts. The method is particularly adaptable for utilising flow cytometry and insoluble chromogenic or fluorescent substrates. The method allows repeated rounds of *in vitro* mutation and recombination between selected mutants, followed by screening for isolates with an altered pH optimum for activity and selection of the most fit survivors. One particular use resulted in the isolation of novel glycosyl hydrolases with superior enzymatic specifications, their expression, and evaluation for their industrial potential, i.e. pH suitability.

The present applicant has also developed a novel method for the *in vitro* evolution of proteins called Degenerate Oligonucleotide Gene Shuffling (DOGS) (WO 02/18629, incorporated herein by reference). The DOGS method was designed for shuffling genes from different families, but it has now been found that it is applicable to random mutations of a single gene so that the limits of sequence space can be explored. This is performed by misincorporation mutagenesis during PCR synthesis of a copy of the target gene. The iterative screening of misincorporation mutagenesis libraries with low mutation frequencies (1-2 amino acid changes/protein) allows the stepwise accumulation of adaptive mutations. Stepwise accumulation of single mutations allows the sampling of a far larger functional sequence space than if highly mutated recombinants are screened. As a consequence, misincorporation mutagenesis tends to select for adaptive mutations while discarding neutral mutations or mutations which decrease the fitness of an enzyme.

Phenotypes such as thermostability can be readily improved by iterative incorporation of single mutations. The present inventors have developed a technique to determine whether a phenotype that is derived from the interaction of multiple amino acids (in this case, an enzyme's pH optimum) might require the accumulation and interaction of neutral mutations (neutral in isolation), and adaptive mutations. A preferred form of the method according to the present invention uses iterative misincorporation mutagenesis in the normal manner.

However, no screening for adaptive mutations occurs or is required. Instead, screening is only done for retained ability (whether unchanged, improved or reduced) to catalyse the hydrolysis of a substrate. All positive recombinants would be combined and used as template for a further round of mutagenesis and so on for as many rounds as are necessary. In this manner, it is assumed that accumulation of multiple adaptive, neutral and harmful (but not inactivating) mutations would occur. Once generated, this library can then be screened for enzymes with modified pH optima, for example. Recombinants with the desired phenotype optionally can then be backcrossed with the parental wild-type gene using DOGS to remove any mutations not contributing to the altered phenotype.

PROCEDURE

Figure 1 shows a schematic of a method for random drift mutagenesis according to the present invention. Step 1 involves mutagenic PCR of a number of copies of a gene encoding an enzyme to form a plurality of mutant genes. The mutated genes are then ligated into plasmids and transformed into *E coli*. The bacteria are then cultured in selective media that contains a substrate which, when acted upon by any functional enzyme in the cell will cause the cell to have a detectable phenotype. The cultured cells are then sorted by a flow cytometer and cells having the phenotype induced by the enzyme are selected. All selected cells will contain a gene encoding a functional enzyme. Plasmid DNA can be obtained from any of the cells and processed again through the method to obtain further mutants. After a number of rounds of mutagenesis and cell selection, individual mutants can be screen for enzymes having a desired or altered characteristic. The mutants can be further mutated or crossed by other means.

EXAMPLES

The experiment uses the gene for a thermostable enzyme previously isolated by us and a relatively inexpensive substrate. The method will also be

applied it to the gene for our *Dictyoglomus* XynB enzyme that is a candidate for commercialisation in the pulp bleaching area (and would use a custom-synthesized substrate in the mutagenesis experiments).

5 Experimental and Techniques

Gene: *Caldicellulosiruptor saccharolyticus* β -glucosidase, *bglA*; Substrate: β -D-chloroindolyl-galactoside (X-gal) or β -D-chloroindolyl-glucose (X-glu). Cleavage of these commercial substrates by hydrolases such as BglA results in the precipitation of insoluble indigo, in our case, inside the cell.

10 Steps:

- (i) PCR mutagenesis of BglA gene to give 1-2 changed amino acids per recombinant.
- (ii) Ligate mutagenic PCR product into vector and transform into Δ ac host.
- (iii) Outgrow transformation mix in LB+X-gal to allow expression of BglA and hydrolysis of X-gal. Sort cells by flow cytometry into those containing indigo (BglA⁺), and those without (BglA⁻).
- (iv) Take BglA⁺ cells, make plasmid DNA.
- (v) Go to step (a). Use as template for next round of mutagenic PCR.
- (vi) At the end of each round, plate a portion of the recombinants, check they are still active (check also ratio of +ve to -ve to confirm sorting procedure) and sequence some recombinants to determine level of accumulation of mutations.

Steps (i) to (vi) should be able to be done in 1 to 2 days. This technique should allow selection from about 10^6 potential mutants at each round of cell sorting. If only 10% of single point mutation mutants retain activity, then potentially 10^5 mutants could be sorted and retained at each round. This method allows the rapid selection of recombinants with multiple mutations and eliminates tedious screening procedures.

Expected outcomes

Potentially any substrate that can accumulate within a cell could be used for sorting. All of the X-substrates such as X-gal and X-glu could be used as they accumulate as indigo within cells. Potentially, we could have X-xylobiose synthesised (X-cellobiose is commercially available so this is technically feasible) to use in xylanase mutagenesis for further improvement of the performance of XynB from *Dictyoglomus*.

10 RESULTS

We have shown that the hydrolysis product of X-gal can be used to sort cells based upon the accumulation of indigo within those cells expressing the BglA+ phenotype. Figure 2 shows the chemical reaction by enzymatic hydrolysis of the chromogenic substrate in the cell causing a color change to the bacterial cell with a functional enzyme.

Figure 3 shows that these indigo-labelled cells can be readily distinguished from unlabelled cells using a Becton Dickinson FACS Calibur Flow cytometry system employing an Argon ion 488 nm laser.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Dated this fourth day of October 2002

Macquarie University

Patent Attorneys for the Applicant:

ALLENS ARTHUR ROBINSON

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Figure 1.

Random drift mutagenesis of hydrolases using a fluorimetric substrate

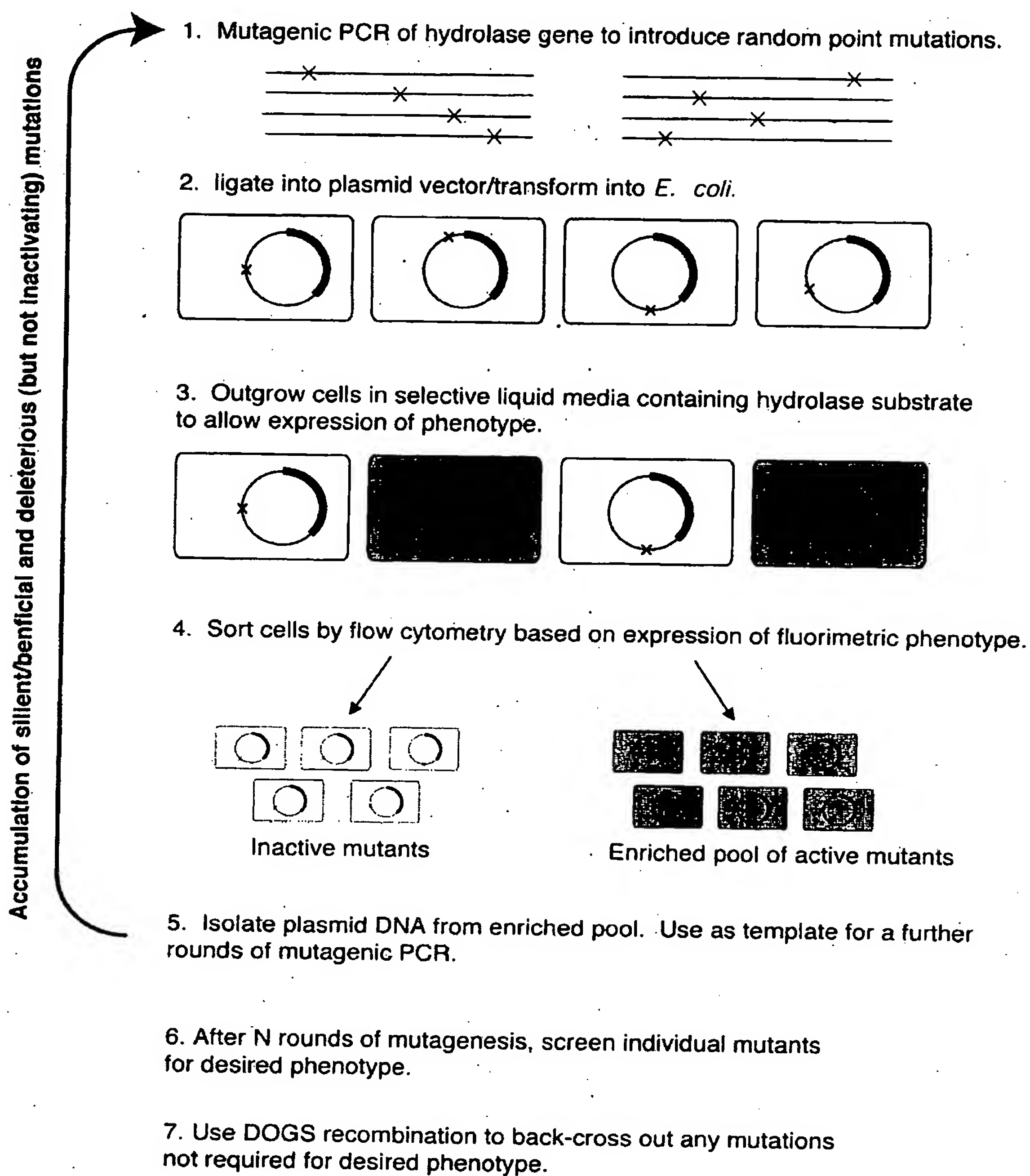


Figure 2.

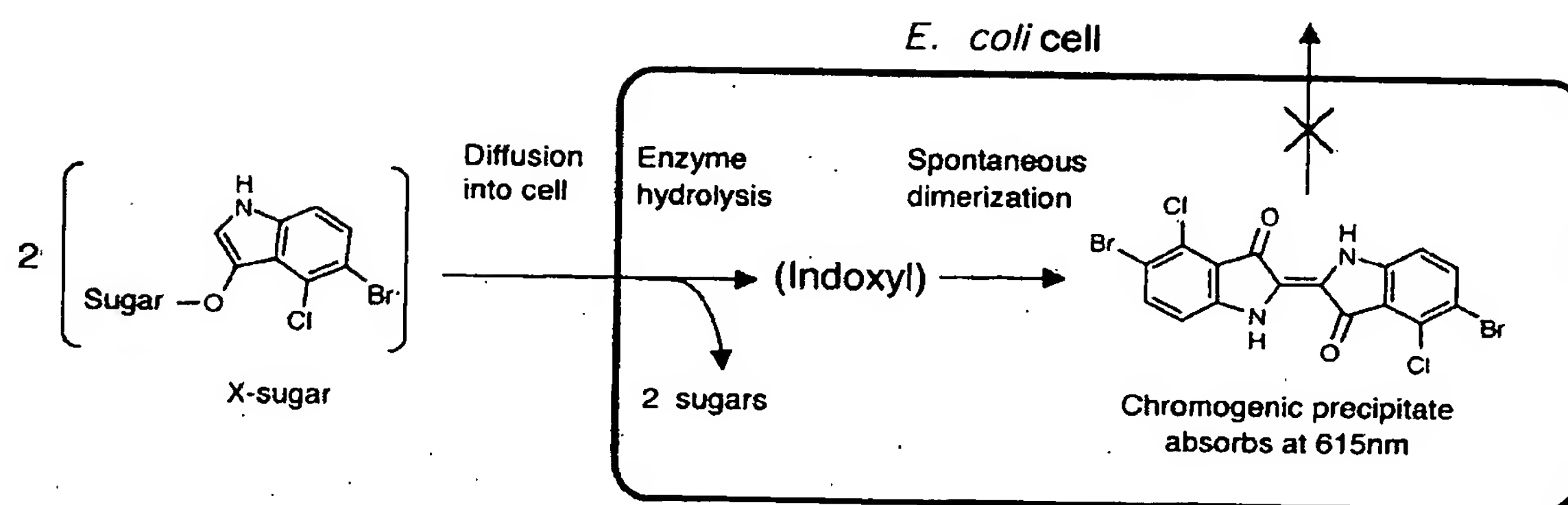
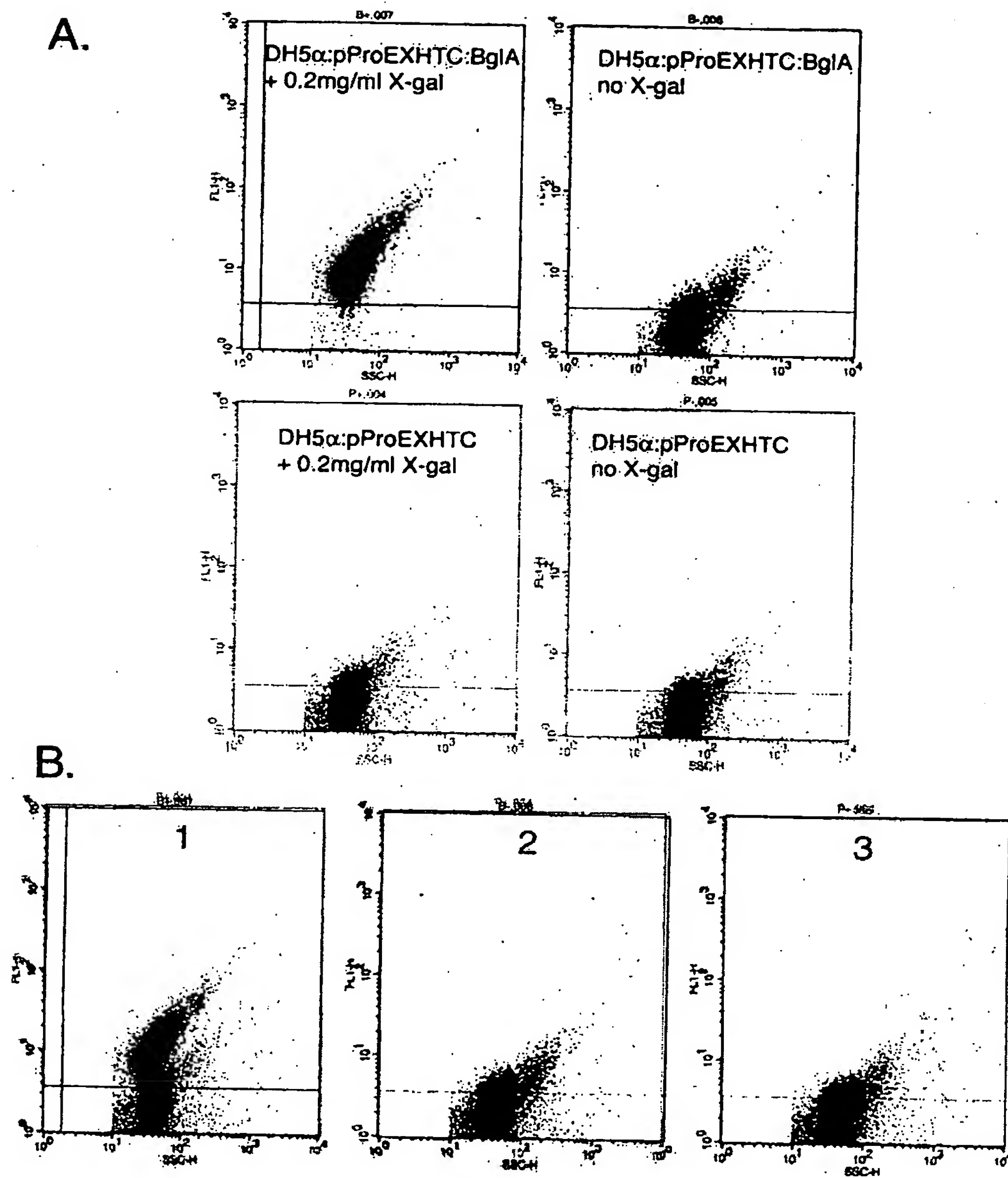


Figure 3.



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